

Determinants of Mucosal HIV Replication and Shedding

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EVER SINCE HIV WAS FIRST DISCOVERED in 1985, the bulk of research conducted has focused primarily on the pathogenesis of this virus in peripheral blood mononuclear cells (PBMCs). However, the mucosal-associated lymphoid tissues (MALT) are the largest source of lymphocytes, macrophages, and dendritic cells in the body, rendering them among the most important—and least understood—repositories of HIV.

The significance of mucosal surfaces in the pathogenesis of HIV cannot be overstated. Mucosal surfaces—including those in the alimentary tract—are an important route by which HIV may gain access to blood and lymphoid tissue during sexual and perinatal transmission. What's more, the mucosa may be involved in the initial selection of viruses that are transmitted to adults and infants and may be a site where virus replication persists and drug-resistant viruses evolve during HAART.

To help make sense of the role of MALT in HIV disease, not only as an entry point for the virus but also as a site of chronic HIV replication, Dr. Brodie took the podium at September's PRN meeting to review preliminary results from two ongoing studies being conducted at the University of Washington in Seattle.

The Big Questions

"ONE ISSUE WE'VE BEEN PARTICULARLY INTERESTED IN IS SUSCEPTIBILITY TO HIV INFECTION through the oral mucosa and whether oral lymphoid tissues provide a reservoir of persistent virus replication during HAART," Dr. Brodie said in providing some background information about his team's work. He then went on to review a study published in a

1996 issue of *Annals of Internal Medicine*, examining the epidemiological correlates of HIV transmission (Schacker, 1996). The study enrolled 46 adults—43 men and three women—with primary HIV infection who presented to the University of Washington clinic, an average of 51 days after HIV seroconversion. Of the 12 patients who could identify the precise date of and activity leading to seroconversion, four reported having only oral-genital contact. "This study raised a lot of questions for us," Dr. Brodie commented. "If oral transmission of HIV occurs, which of the diverse epithelial structures in the oral cavity facilitate transmission of the virus? And, what are anatomic sites and cells that support virus replication?" Alternatively, if oral transmission occurs, is it bidirectional? In other words, are both the insertive and receptive partners of oral sex at risk for HIV infection?

Another important question involves drug-resistant HIV. There is no shortage of clinical trial data or real-world experience demonstrating that a significant proportion of HIV-positive individuals do not fully benefit from antiretroviral treatment because of drug-resistant virus. Once present, whether acquired at the time of primary infection or selected by antiretroviral therapy, drug-resistant virus usually persists, often limiting the efficacy of subsequent treatments because of cross-resistance between most available drugs. "Our knowledge regarding the establishment and persistence of reservoirs of drug resistant virus is limited," Dr. Brodie pointed out. "However, it is these reservoirs that need to be understood and overcome for successful treatment of a large segment of the population of infected individuals. Hence, our studies have focused on determining the distribution of drug-resistant

viruses in different cell types within peripheral blood, oropharynx and rectal mucosa, and how these populations wax and wane over time and with treatment."

Together, Dr. Brodie speculated, "These detailed studies may provide insight needed to improve strategies to diagnose and treat drug-resistant infections, including ways to better target therapies to mucosal surfaces."

HIV in Oral Mucosa

MUCH OF WHAT IS KNOWN ABOUT HIV REPLICATION in mucosal tissues comes from studies involving the male and female reproductive tracts and gut-associated lymphoid tissues. Hence, "one of the first things we wanted to look for was HIV replication in oropharyngeal lymphoid tissues to see if there were differences in virus-cell tropisms and to assess the relative amount of viral gene diversity in these tissues," Dr. Brodie explained.

First up was a preliminary study, in which 70 sexually active HIV-positive gay and bisexual men attending clinics in Seattle and Lima, Peru, were enrolled. Dacron and other types of absorbent swabs were used to collect mucosal secretion samples from the oral cavity. It turned out that approximately one-third of the patients had evidence of HIV-RNA in the oropharyngeal swabs, a finding that was independent of HAART. Most of the HIV-RNA produced was in the distal pharynx, in areas of palatine tonsils. Dr. Brodie also pointed out that approximately 25% of the HIV-RNA-positive swabs contained infectious virus.

None of the whole saliva samples from the 70 study volunteers produced HIV-RNA or contained infectious virus. "But there

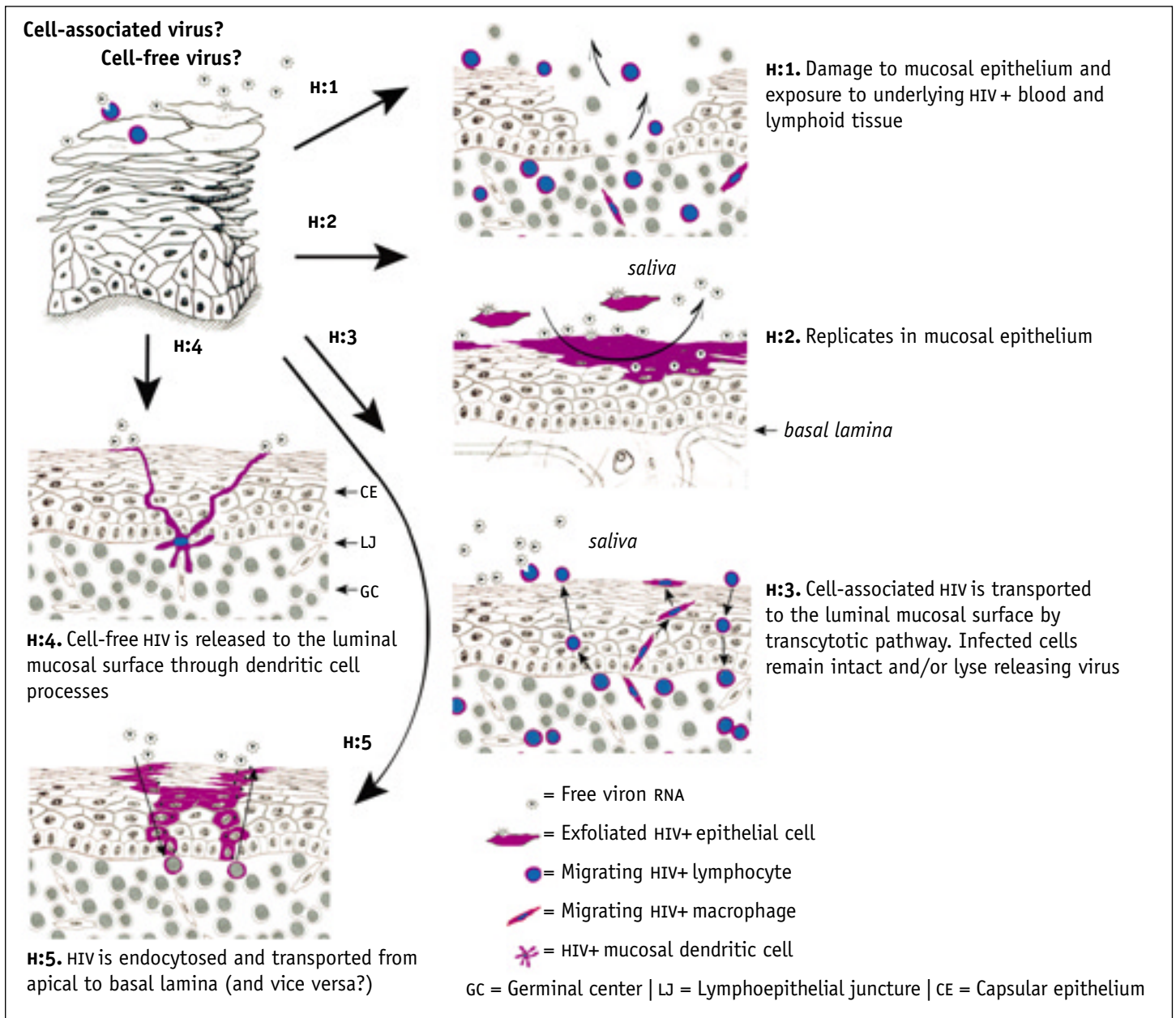


FIGURE 1. Hypotheses of Oral HIV Expression

Hypothetical representation of HIV infection and replication in oropharyngeal mucosa.

Source: Scott Brodie, DVM, PhD

are reports of individuals who are hyper-secreters who do have high concentrations of infectious HIV in saliva,” Dr. Brodie said. “It’s very likely that these individuals may have a protease or other enzymatic defect in their saliva, which may increase the risk of transmission via saliva.” However, this is thought to be a very rare condition.

The isolation of HIV in the oral mucosa of a sizeable percentage of HIV-positive patients was intriguing enough to warrant a more complex study. “Our first aim in developing another study was to define the anatomic sites of HIV replication in the

oropharynx and to determine if the frequency and titer of HIV expressed in oral mucosa is influenced by the presence and proximity of HIV infection in the underlying tissues,” Dr. Brodie explained. The study team’s hypothesis here was that tonsils are the primary sites of HIV replication and the principal source of virus shed from the oral mucosa. HIV-positive patients who have high concentrations of viral RNA in mucosal swabs should, in essence, have higher numbers of HIV-RNA-positive leukocytes in the tonsillar mucosa than patients with no or low levels of oral HIV shedding.

“Finding HIV-RNA-positive cells in the epithelial mucosa of the tonsil led us to conclude that transcytosis of viral RNA-positive cells to the mucosal surface is at least one mechanism accounting for the high titers of HIV in mucosal fluids.”

Another aim of more advanced studies was to determine the range of HIV-permissive host cells in oropharyngeal tissues during primary and progressive HIV infection. “Here we hypothesized that CD4+ lymphocytes and/or dendritic cells are the initiating targets of HIV infection in tonsillar mucosa,” Dr. Brodie said. “CD4+ cells

comprise the majority of early-infected HIV-RNA-positive cells. However, with progression of infection, there are increased numbers of viral RNA-positive macrophages. In turn, we expected to find high-frequency shedders to have proportionately more viral RNA-positive macrophages with higher intracellular viral load than low shedders or nonshedders.” Using a tonsil explant model to study primary HIV infection, Dr. Brodie’s group, in fact, showed that both intraepithelial lymphocytes and dendritic cells—cells migrating within the epithelial mucosa—harbored HIV-RNA. In turn, tonsillar biopsies from persons with chronic HIV infection tended to have the highest numbers of HIV+ macrophages. Persons that shed HIV-RNA had significantly higher intracellular copies of HIV-RNA than nonshedders.

A third aim was to assess HIV population dynamics in the oropharynx. Dr. Brodie’s group hypothesized that the oropharynx is a distinct and separate compartment with populations of virus that are distinct from those found in the blood. “It’s likely that HIV replication in tonsils, measured by the temporal accumulation of mutations in proviral DNA, contributes to the overall sequence diversity in blood plasma and proceeds even when plasma viral load is below thresholds of detection,” Dr. Brodie suggested. “We were not surprised to find that tonsillar macrophages are a significant reservoir of replicating virus during antiretroviral therapy and appeared to accumulate viruses from other tissues, including viruses found in blood.”

A hypothetical representation of HIV infection and replication in oropharyngeal mucosa is highlighted in Figure 1.

At the time of Dr. Brodie’s PRN presentation, approximately 40 HIV-positive patients had been enrolled into the University of Washington’s more comprehensive study. As people enter the study, peripheral blood samples are collected and a variety of serologies are performed. Saliva samples are also collected every day for a period of 31 days. Oropharyngeal swabs—including those of the lingual tonsil, palatine tonsil, hard palate, soft palate, minor salivary gland, and buccal mucosa—are collected on a weekly basis. Finally, on the 31st day of the study, biopsies are taken from the palatine tonsil, the lingual tonsil, and the buccal mucosa.

In reviewing the results of this study,

TABLE 1. Relationship Between HIV Mucosal and Tissue Titers

| Patient | Mucosal Site | Mucosal Virus | | Tissue Virus | |
|--|--------------|----------------------|----------------------------|-------------------------|--|
| | | vRNA (log copies/mL) | Infectious HIV (log IU/mL) | vRNA (log/μg of tissue) | vRNA+ cells per mm ² tissue)* |
| 3 | Lingual | 0.46 | 0 | 1.12 | 0 |
| 4 | Lingual | 1.56 | ND | 1.95 | 7 ± 2.1 |
| 6 | Lingual | 0.89 | 0.02 | 1.02 | 4 ± 1.3 |
| 1 | Pharynx | 5.31 | 0.75 | 6.11 | 33 ± 4.2 |
| 2 | Pharynx | 3.17 | 0.25 | 3.83 | 21 ± 3.1 |
| 4 | Pharynx | 6.13 | ND | 7.10 | 41 ± 5.6 |
| *Mean vRNA+ cells per five consecutive microscopic fields. | | | | | |
| Source: Scott Brodie, DVM, PhD | | | | | |

which are being prepared for publication in a peer-reviewed journal, Dr. Brodie explained that only swabs from the caudal-lateral lingual and distal pharyngeal mucosa produced HIV-RNA (see Table 1). These are two sites that are rich in lymphoid tissue. “Two- to twelvefold more virus was detected in association with pharyngeal versus lingual mucosa,” he said. HIV-RNA could not be detected in the buccal mucosa, anterior lingual mucosa, rostral pharynx, or the hard or soft palate.

As in the pilot study, a proportion of pharyngeal HIV-RNA was infectious in cell culture (approximately 25% of viral RNA-positive swabs). And no patients in the study thus far have produced whole saliva samples containing HIV-RNA or infectious virus.

With respect to tonsillar involvement, Dr. Brodie explained that the palatine tonsil is frequently found to have a high concentration of HIV-RNA, a significant proportion of which is infectious in cell culture. Not surprisingly, individuals who had tonsillectomies had a significant reduction—more than a half-log drop—in pharyngeal HIV-RNA titers. Tonsillar CD4+ lymphocytes were the most abundant HIV-DNA-positive cell, compared to macrophages and dendritic cells. However, lymphocytes and macrophages produced the vast majority of HIV-RNA—cells that were observed adjacent to and within layers of the epithelium of the tonsils.

“It’s important to recognize that the epithelium of the palatine tonsil pillars can be relatively thin-layered and a common site of necrotizing inflammation, compared to the epithelium of the anterior pharynx and

buccal mucosa, which are six- to tenfold greater in thickness,” Dr. Brodie pointed out. “Where there has been some erosion or ulceration of the palatine tonsil, you can find HIV-positive lymphocytes right on the surface.” This, Dr. Brodie implied, could contribute to high HIV-RNA titers in mucosal secretions and may have significant implications with respect to the potential for oral spread of HIV.

The lingual tonsil also harbored HIV-RNA-positive cells, a proportion of which were macrophages. “We also observed that the number of cells expressing the HIV coreceptor CCR5 and tissue concentrations of CCR5 mRNA were increased in tissues from shedders compared to nonshedders and persons with inflammatory lesions of the pharynx,” Dr. Brodie added. “Viral RNA-positive dendritic cells expressing the HIV binding lectin, DC-SIGN, were observed migrating within tonsil epithelium.” It is unclear whether these cells replicate HIV or just bind virus via cell-surface receptors from which they can then transport HIV to organized lymphoid tissues where there is an abundance of virus-permissive cells.

While CD4+ lymphocytes were the most frequently found to be infected with HIV, tonsillar macrophages harbored more HIV-RNA than lymphocytes on a per-cell basis, and produced proportionally more multiply spliced HIV-RNA—i.e., greater amounts of potentially infectious HIV. Interestingly, there were increased numbers of HIV-RNA-positive macrophages and fewer HIV-RNA-positive CD4+ cells with progressive disease. Dr. Brodie suggested that this may have resulted from a decrease in the “pre-

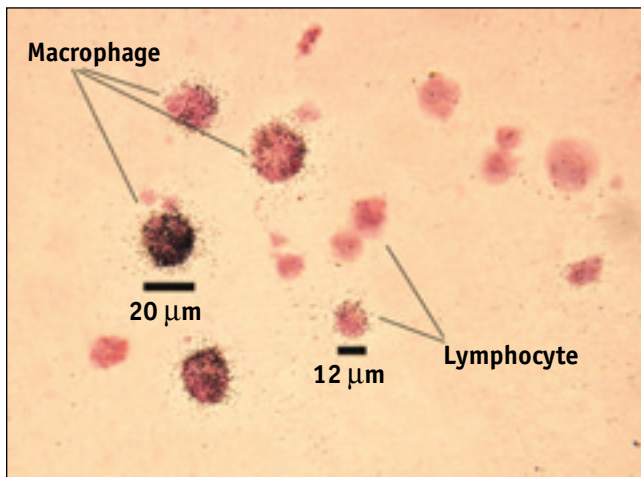


FIGURE 2. Rectal Macrophages Versus Lymphocytes
 Localization of HIV-RNA in mononuclear cells obtained by swabbing the rectal mucosa. By counting silver grains, where one silver grain is equivalent to about one virus copy, macrophages contained more HIV-RNA than lymphocytes on a per-cell basis (*in situ* hybridization).

Source: Scott Brodie, DVM, PhD

ferred substrate” for HIV or gradual adaptation of virus to other cell types.

Finally, phylogenetic analyses demonstrated that tonsils were a distinct and separate virologic compartment from the population of virus in blood, and that macrophages were the most significant contributor to unique viral populations. Viruses derived from tonsillar biopsies from patients treated with HAART showed greater genetic diversity and divergence than viruses obtained simultaneously from blood, suggesting that the effective population size of virus in tonsil was greater than that in blood. “At present,” Dr. Brodie commented, “we have no evidence that drug-resistant viruses evolve more rapidly in tonsil. But, the jury is still out.”

HIV in Rectal Mucosa

AS IS REVIEWED IN “HIV AND THE GUT: FEEDING the Enemy,” an article appearing in the September 2000 issue of *The PRN Notebook*, the intestine comprises approximately 40% of the body’s total lymphoid mass and more than half of its mucosal surface area. Beyond data indicating that it is an early site of both HIV infection and immune cell depletion, there is no shortage of epidemiological evidence implicating contact with anorectal secretions as a major risk for transmitting HIV. However, there has been little information regarding

the presence of HIV in rectal lymphoid tissue and its relationship to mucosal shedding of HIV. In turn, a research team at the University of Washington—with Dr. Brodie at the helm—conducted a study to determine if there was an association between HIV viral load in rectal lymphoid tissue and mucosal shedding of HIV-positive men, including those receiving antiretroviral therapy. A comprehensive review of this study has been submitted for publication.

As in the oropharyngeal study, the University of Washington group enrolled patients through their own clinic in Seattle

and in collaboration with the University of Cayetano Heredia in Lima. Twenty-four men, all of whom reported ongoing anal-receptive intercourse with other HIV-positive men, agreed to participate. Twelve HIV-negative age-matched gay and bisexual men served as controls.

Upon obtaining rectal secretions and biopsies of rectal mucosa, quantitative PCR, *in situ* PCR, and *in situ* RNA hybridization assays were used in combination with immunocytochemistry to identify sites of HIV replication. Phylogenetic analyses were also performed to evaluate if viruses from rectal mucosa evolved distinct from viruses in blood.

Among the 24 men studied, HIV-RNA was detected in rectal secretions from six (25%), including one of three patients not treated with antiretroviral therapy, five of 21 antiretroviral-treated patients, and two of seven patients with HIV-RNA levels below 200 copies/mL in blood plasma (see Table 2). In fact, 4/6 (67%) men who shed rectal HIV-RNA versus 13/18 (72%) nonshedders had detectable HIV-RNA in plasma at the time of enrollment and sample collection. Among the six men from whom HIV was detected in rectal secretions, the titer of HIV-RNA in rectal secretions was eightfold higher than that found in peripheral blood plasma in five of these men.

With respect to HIV in biopsied rectal tissues, Dr Brodie explained that men shedding HIV had higher tissue concen-

trations of HIV-RNA in biopsy samples than nonshedders. Also of interest, infectious virus was isolated from rectal tissues of all men found to be shedding HIV in their rectal secretions, compared to less than 40% of nonshedders.

All of the men had evidence of HIV-DNA in rectal tissues, but there were no discernible differences in tissue concentrations of HIV-DNA-positive cell counts between shedders and nonshedders. This suggests that many individuals are covertly infected and may potentially shed virus when circumstances allow for virus replication to become active. “Such ‘activation signals’ may involve coinfection with other sexually transmitted infections,” Dr. Brodie added.

While there was no correlation between plasma viral load and rectal concentrations of HIV-DNA, HIV-RNA shedding in secretions was proportional to the number and type of HIV-RNA-positive cells in the underlying mucosa. High concentrations of rectal HIV-RNA correlated with higher numbers of mucosal HIV-RNA-positive lymphocytes and macrophages (see Figure 2). Interestingly, men who shed greater than 100,000 HIV-RNA copies/mL of rectal secretion had proportionally more HIV-RNA-positive macrophages in the mucosa than men who shed less than 20,000 copies/mL. “A possible explanation for this,” commented Dr. Brodie, “is that macrophages are less permissive to certain antiretroviral drugs. For example, protease inhibitors are reported to be less effective in macrophages than in lymphocytes.”

The importance of HIV replication in rectal lymphocytes and macrophages was underscored by the genotypic analyses of the HIV population dynamics in the rectal samples. During antiretroviral treatment, rectal mucosa had, approximately, a 1.3-fold larger effective viral population size than the population found in PBMCs. “This tells us that either more HIV-producing cells were populating in the rectal mucosa or that HIV-producing cells in the rectal mucosa were manufacturing more virus than cells seeding in the blood,” Dr. Brodie said. “In other words, viruses in the rectal mucosa appeared to replicate independently of viruses in the blood, suggesting that the rectal mucosa is a distinct virologic compartment, in ways similar to what we’ve been seeing in our oropharyngeal study.”

A totally unexpected finding of this study was the presence of appreciable stores of

